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Strong Activation of Parallel Fibers Produces Localized Calcium Transients and a Form of LTD That Spreads to Distant Synapses

Nick A. Hartell*
Laboratory for Synaptic Function
Frontier Research Program
RIKEN
Wako-shi, Saitama 351-01
Japan

Summary

The temporal and spatial changes in intracellular calcium levels during separate activation of parallel fiber (PF) and climbing fiber (CF) inputs to cerebellar Purkinje cells were studied. PF stimulation (1 Hz), at relatively high stimulus strengths, led to accumulations of calcium that were similar in peak levels to those following CF stimulation but that remained spatially localized. Such stimuli consistently induced a durable depression of PF synaptic transmission that partially occluded further depression by conventional conjunctive stimuli and that was independent of nitric oxide. This depression was accompanied by a reduction of synaptic efficacy in spatially isolated PF inputs to the same cell that was independent of postsynaptic calcium but that was mediated by nitric oxide. These data indicate that LTD comprises at least two separate processes and that parameters of PF stimulation that are capable of raising calcium levels in Purkinje cell dendrites are also able to induce long-term changes in synaptic efficacy.

Introduction

Simultaneous, repetitive activation of parallel fibers (PFs), the axons of cerebellar granule cells, and climbing fibers (CFs), axons of inferior olivary neurons, leads to a long-term depression (LTD) of transmission at the PF–Purkinje cell synapse (Ito et al., 1982). Three basic elements are required for LTD: activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Linden et al., 1993; Hemart et al., 1995), which mediate fast transmission at the PF–Purkinje cell synapse (Hirano, 1990; Konnerth et al., 1990; Ajima et al., 1991); activation of metabotropic glutamate receptors (Linden et al., 1991; Hartell, 1994a; Shigemoto et al., 1994; Alba et al., 1994; Conquet et al., 1994), which are likely to be activated at the rates of PF stimulation necessary for LTD induction (Batchelar and Garthwaite, 1993); influx of calcium via voltage-gated calcium channels. Microfluorometric observations reveal that CF stimulation evokes large increases in calcium in Purkinje cells (Ross and Werman, 1987; Sugimori and Llinas, 1990; Konnerth et al., 1992). This synaptic requirement for LTD can be replaced either by cell depolarization to levels sufficient to allow calcium influx through voltage-gated calcium channels (Crepel and Krupa, 1988; Hirano, 1990) or by release of caged calcium (Kasono and Hirano, 1994). Furthermore, LTD is prevented by the introduction of calcium chelators into Purkinje cells (Sakurai, 1990; Linden and Connor, 1991; Shibuki and Okada, 1992; Konnerth et al., 1992).

Recent evidence has shown that PF stimulation can also cause significant calcium influx, but in highly localized branchlets within the Purkinje cell dendritic arborization, simply by the opening of voltage-dependent calcium channels following activation of AMPA receptors (Eilers et al., 1995). This implies that PF stimulation alone can potentially satisfy the three basic requirements for LTD and challenges current views of cerebellar learning and memory based on the Marr–Albus hypothesis (Marr, 1969; Albus, 1971), which predicts that CF activity (and, by inference, calcium influx) serves as an error detection mechanism that functions through LTD to weaken selectively those PF inputs that are activated simultaneously. Notably, however, PF stimulation alone has not been reported to induce LTD (for reviews, see Ito, 1989; Linden, 1994a).

To examine whether PF stimulation is capable of inducing LTD, we used confocal microscopic fluorimetry, combined with whole-cell recording, to compare the size and duration of PF- and CF-mediated calcium responses in identical regions of the same cells. Parameters of PF stimulation that led to accumulations of calcium that were similar in peak size to those resulting from CF stimulation consistently induced a novel form of synaptic depression (LTD_{PF}). This depression was dependent upon postsynaptic calcium but did not require nitric oxide (NO). LTD_{PF} was also accompanied by a robust depression of synaptic transmission in spatially and synaptically discrete PF pathways to the same cell that was independent of postsynaptic calcium but which was mediated by NO. These results demonstrate that repetitive activation of PFs can mobilize sufficient calcium to induce LTD at the PF–Purkinje cell synapse. Furthermore, LTD appears to comprise at least two separate processes: one that is calcium dependent and the other being mediated by the diffusible messenger NO. This finding that PF stimulation alone can induce LTD if PF excitatory postsynaptic potentials (EPSPs) are larger than a threshold value of 8–10 mV adds a novel mechanism to the function of cerebellar cortical networks.

Results

Comparison of PF- and CF-Mediated Calcium Influx

In Purkinje cells loaded with the fluorescent calcium indicator dye calcium green-1, strong stimulation of PFs resulted in clear elevations in fluorescence intensity in highly discrete regions of the dendritic tree comprising one or more branchlets (Figure 1). Calcium transients were most easily observed in cells held under current–clamp at potentials less negative than −70 mV, but they

*Present address: Department of Pharmaceutical and Biological Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom.

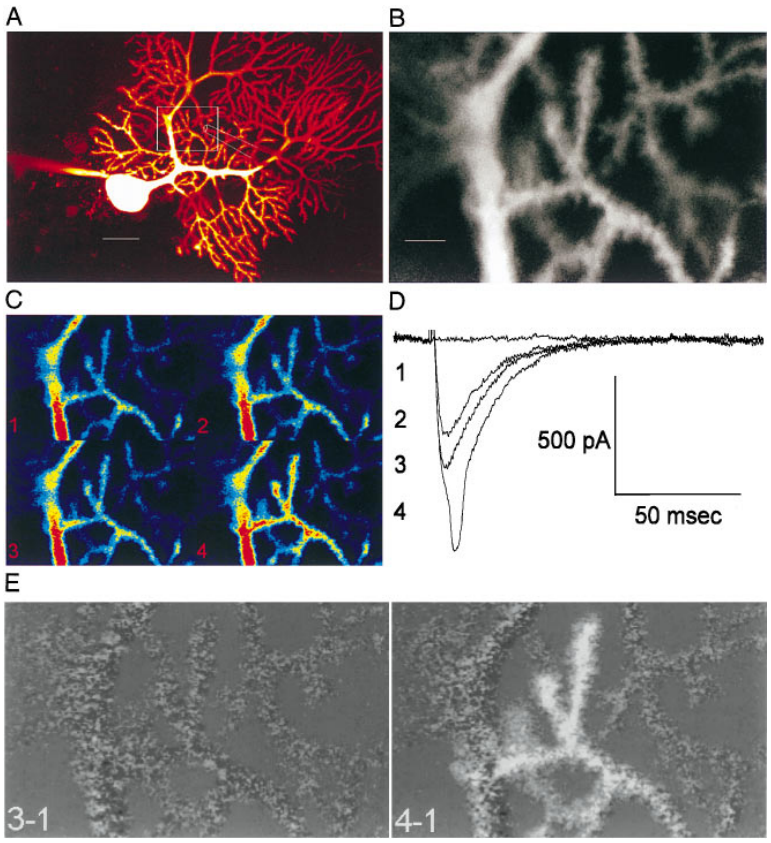

Figure 1. PF-Mediated Calcium Influx

(A) Purkinje cell filled with 1 mM calcium green-1. The position of one of two stimulating electrodes is outlined. Scale bar represents 25 μm.

(B) Magnified view of the region outlined in (A) (scale bar, 5 μm).

(C) Images 1–4 are single, pseudocolor images, illustrating the effects of increasing stimulus strength, whose collection was triggered to coincide with PF stimulation at a range of intensities. Black–blue–yellow–red indicates lowest to highest fluorescence intensity. The time taken for a complete scan was approximately 170 ms.

(D) Simultaneously recorded whole-cell currents. The lowest current trace shows an inflection that can probably be ascribed to opening of voltage-dependent calcium channels by an adequate local depolarization.

(E) comprises a subtraction of image 1 from 3 and 4, taken from (C).

could also be seen under apparent voltage–clamp conditions at stimulus intensities below those leading to the appearance of regenerative spikes (Figures 1C and 1D). In agreement with Eilers et al. (1995), both spines and their supporting dendrites showed increases in calcium concentrations (Figure 1E). Although no evidence for calcium changes restricted only to spines was obtained at lower stimulus intensities, this may well reflect limitations of temporal and spatial resolution in the measurement system used. Line-scanning techniques (see Figure 2 and Experimental Procedures) confirmed that PF-mediated calcium responses were not only spatially confined, but also graded with stimulus intensity once a threshold EPSP amplitude of approximately 8–12 mV was exceeded (mean value 9.4 mV, n = 6; for an example, see Figure 2). The size of PF-mediated calcium transients, elicited at constant stimulus intensities, was strongly dependent on the membrane potential, indicating that these transients occur through a voltage-dependent mechanism and confirming the results of Eilers et al. (1995).

Data obtained from precisely the same regions of the same cells following separate activation of PFs and CFs revealed that peak PF-mediated calcium transients were, depending on the stimulus intensity, potentially as large and occasionally larger than those produced by CF stimulation. CF-mediated calcium transients were all or none and occurred in both dendrites and somata. A typical example is shown in Figure 3. Although peak calcium levels were similar, the durations of PF responses were consistently shorter. The recovery phase was best fitted with a double exponential curve with mean decay time constants of 184 ± 68 and 524 ± 59 ms (mean ± SEM; n = 10). The decay of fluorescence following CF stimulation was significantly longer ($p < 0.05$; two-tailed Student's t test) with mean time constants of 212 ± 67 and 2251 ± 704 ms (n = 6). For both synaptic inputs, the durations of the calcium responses far exceeded those of their EPSPs (134 ± 9 ms [n = 11] and 188 ± 72 ms [n = 4] for PF and CF, respectively). Although the kinetic properties of calcium green-1 will cause an overestimation of the length of calcium transients, this cannot account for the significantly longer duration of calcium transients observed following CF stimulation. At intensities of stimulation for which the peak PF and CF calcium responses in the same cell were similarly matched, the integral of δF/F for the 3 s measurement period revealed that CF stimulation was up to 60% (mean = 47%; n = 4) more effective in elevating calcium than PF stimulation, owing to the slower return to baseline levels. Repetitive stimulation of either input at 1 Hz resulted in a progressive accumulation of intracellular calcium. Although similar peak increases were observed (Figure 3B), the overall degree and duration of calcium elevation following CF stimulation was consistently greater (n = 5).

PF-Mediated Calcium Influx Is Sufficient to Induce LTD?

Those parameters of PF stimulation that were most effective in raising intracellular calcium were tested on PF inputs to Purkinje cells to determine whether this was

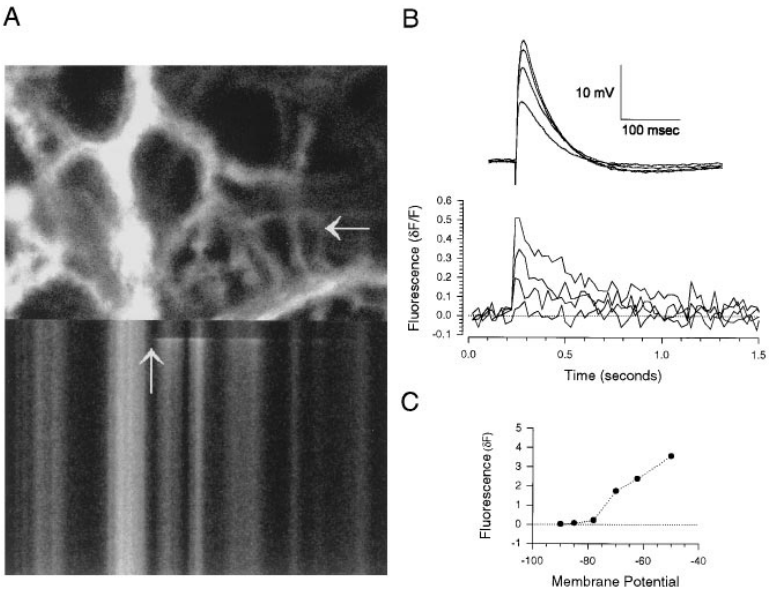

Figure 2. PF-Mediated Calcium Responses Are Graded with Stimulus Intensity

Data shown in (A) and (B) were obtained from a Purkinje cell current–clamped to −70 mV in normal medium.

(A) Top: the position for single line scanning of a dendritic region is indicated by the horizontal arrow. Bottom: 126 successive line scans taken at 24 ms intervals (top to bottom time shown was approximately 3 s). A single PF stimulus was given shortly after scan onset. Only regions to the right of the vertical arrow responded to PF stimulation and hence were averaged separately.

(B) Increasing stimulus intensity increases EPSP amplitude (upper traces) and fluorescence intensity and decay time (lower traces).

(C) Effects of changing membrane holding potential on PF evoked peak fluorescence responses to constant intensity stimulus pulses.

sufficient to induce synaptic depression. In cells clamped at membrane potentials between −70 and −65 mV, two separate PF pathways, test (PF_1) and control (PF_2), were activated alternately at a rate of 0.2 Hz (see Experimental Procedures for details). Control stimulus intensities were chosen to induce EPSPs of amplitudes between 4–6 mV to minimize calcium entry. In the majority of experiments, constant current stimuli (range 5–30 μA) were used. In a few cases, constant voltage stimuli were applied (5–15 V). After a suitable control period, the intensity of stimulation to only the test input (PF_1) was raised to elicit EPSPs of amplitudes above 10 mV, and the frequency of stimulation was simultaneously increased to 1 Hz. Immediately following the change in stimulus parameters, a progressive depression of EPSP slope from the new peak level was observed (Figure 4A, n = 7, closed circles). Depression was rapid and could be observed within the first two or three responses and reached near maximal levels within 10 min. Once depression was induced, halting the stimulus to the test pathway for 5 min did not lead to any subsequent recovery of responses elicited by these same, raised parameters of stimulation.

To determine whether the depression observed in PF_1 was dependent on the continued, raised level of stimulus intensity, a limited duration induction protocol was used.

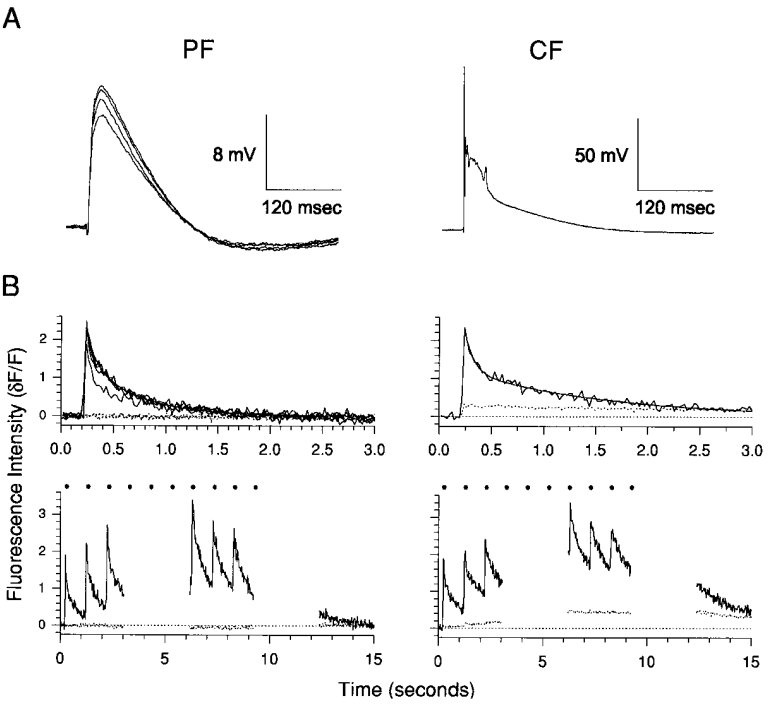

Figure 3. Comparison of PF- and CF-Mediated Fluorescence Responses

Fluorescence changes following PF (left) and CF stimulation (right). The results shown here are from the same cell illustrated in Figure 2A. The solid lines are the average of regions to the right of the vertical arrow in Figure 2A. The dotted trace is the average for the regions to the left of the vertical arrow. The bottom panel illustrates shifts in the basal levels of the fluorescence responses with 10, stimulus pulses at 1 Hz to PF and CF inputs. Responses of the region to the left of the vertical arrow are shown dotted. The dots above the graph indicate the timing of the stimuli.

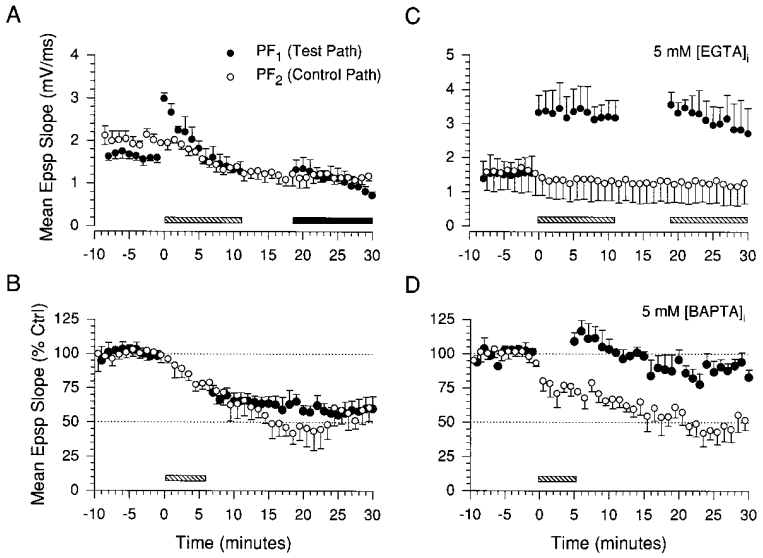

Figure 4. PF-Mediated LTD

(A) The effects of a sustained increase in stimulus intensity and frequency to one of two separate PF pathways (PF_1, closed circles). Responses to PF_2 (open circles) were elicited at constant stimulus intensity and at 0.2 Hz throughout. Means and standard errors of EPSP slopes are plotted against time (n = 4). The hatched bars indicate the periods during which the intensity and frequency of stimulation PF_1 were raised. Stimulation to PF_1 was halted for the period between the hatched bars.

(B) The effects of 5 min of 1 Hz high intensity stimulation to PF_1 (n = 5). After 5 min, stimuli were resumed at pretest, control levels. For clarity, the PF_1 responses evoked in during the test stimuli are omitted. No changes in stimulus parameters were made to PF_2 (open circles). Data are expressed as percentages of control responses.

Experiments outlined in (A) and (B) were repeated with 5 mM EGTA (C, n = 4) and 5 mM BAPTA (D, n = 5) in the recording pipette.

Experiments were repeated as above, but the test stimulus to PF_1 was applied for only 5 min. Stimulation was then resumed at the original frequency and intensity. Figure 4B shows that this brief test stimulus was sufficient to induce a sustained depression of PF responses to levels well below control (n = 5). Since the level of depression induced by this method, relative to control values, was statistically indistinguishable from that induced by the continuous stimulus protocol (normalized to the peak level at the onset of the induction stimulus paradigm), data from both experimental groups were pooled together and summarized in Figure 5A.

Inclusion of either 5 mM EGTA (n = 5) or 5 mM BAPTA (n = 5) to the recording pipette entirely prevented depression of PF_1 responses for both the continuous and 5 min induction protocols, respectively (Figures 4C and 4D, closed circles). With standard internal solutions, simply raising the stimulus frequency to 1 Hz but maintaining the EPSP amplitudes below 7 mV had little or no effect on PF_1 responses (n = 5). Increasing just the stimulus strength led to a partial depression (n = 6) that was also blocked by EGTA (n = 5). These data are summarized in Figure 5A.

Figure 5B illustrates the dependence of this PF-mediated LTD (LTD_{PF}) on a threshold level of evoked EPSP amplitude. In this experimental group, after a 10 min control period, the frequency of stimulation PF_1 was raised to 1 Hz and the stimulus intensity was increased to produce initial responses of peak amplitudes anywhere between 4 and 13 mV. The extent of the resulting depression of EPSP slopes, normalized to the transient peak level following the change in stimulus parameters, was plotted against the peak EPSP amplitude (n = 17). (EPSP amplitude was used in preference to absolute stimulus current, since this latter parameter was strongly dependent upon the position of the electrode and hence variable between slices. There was, however, no indication that the size of the stimulus current between experiments influenced either the time course of similarly matched amplitude EPSPs or their susceptibility to depression.) At peak amplitudes less than approximately 8 mV, only 1 out of 7 cells underwent depression to a level more than 80% of the peak response. All 10 cells whose initial EPSP amplitudes exceeded 9 mV underwent depression to more than 80% of the peak value. This threshold level correlates reasonably well with that for PF-mediated calcium influx (9.4 mV) and indicates that stimulation of PF inputs at 1 Hz and at intensities that produce EPSPs of over 8 mV results in sufficient calcium influx to cause a robust depression of synaptic transmission.

LTD_{PF} Is Accompanied by a Heterosynaptic Depression of Spatially Discrete PF Inputs

As is apparent from Figures 4 and 5, both the continuous and 5 min paradigms of LTD_{PF} induction also caused a clear and robust depression in the control pathway (PF_2), even though the stimulating electrodes were separated by 40–100 μm and the stimulation parameters to this input were not altered. The most obvious explanation for this is that the high intensities of stimulation that were required to induce LTD at PF_1 simply recruited fibers to PF_2 and that depression was then induced in these common inputs. Several lines of evidence suggest that this was not the case. Paired stimuli applied at 30 ms delay to PF_1 led to a facilitation of the second PF_1 EPSP (Figure 6A). This paired pulse facilitation is generally taken to be an indicator of presynaptic release probability, owing to a presynaptic accumulation of calcium. If the second pulse was presented at the same latency but to the alternate input pathway (PF_2), no potentiation of the second EPSP was observed, even at strengths of stimuli that induced PF_1 EPSPs of 16 mV amplitude (Figure 6B). The responses to the second pulse delivered at constant intensity to PF_2 are shown superimposed in Figure 6C. Plotting the normalized slope of the second response (PF_2) against the amplitude of the first (PF_1) confirmed that the response of PF_2 was independent from that of PF_1, irrespective of the stimulus intensity (Figure 6D). The slight depression observed at higher intensities of stimulation is presumably due to the decreasing membrane potential upon which the second

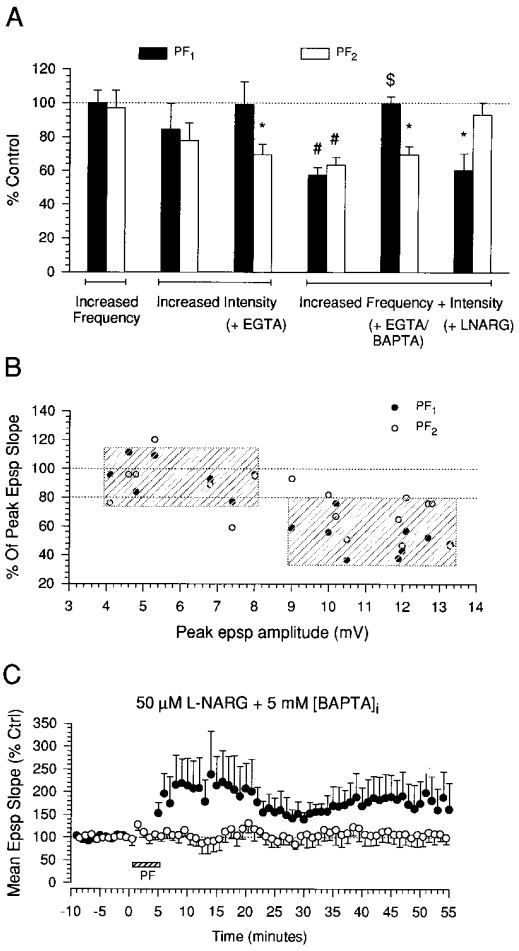

Figure 5. Requirements for LTD_{PF}

(A) Summary of the extent of depression of PF_1 (closed bars) and PF_2 responses (open bars) following a range of stimulus conditions applied to PF_1. These were, from left to right, increasing frequency of stimulation alone; increasing intensity of stimulation alone (and in the presence of 5 mM EGTA); increasing both the frequency and intensity of stimulation to the test pathway in the absence and presence of EGTA and with 20 μM LNARG in the perfusate. Data are expressed either as a percentage of the peak EPSP slope immediately following the change in stimulus parameters or as a percentage of control, depending on whether a continuous or 5 min induction protocol was used. Asterisks indicate where a significant difference was observed between responses in test and control pathways ($p < 0.01$). Hash marks indicate a significant difference compared to the effects of raising just the frequency of stimulation ($p < 0.01$), and dollar signs illustrate a difference relative to the effect of elevating both the frequency and intensity of stimulation in the absence of either EGTA or LNARG (Mann–Whitney U test, $p < 0.05$).
(B) The relationship between the extent of depression of PF_1 (closed circles) and PF_2 (open circles) EPSP slopes at 10 min and the peak amplitude of the PF_1 response measured at the onset of the change in stimulus frequency and intensity.
(C) PF_1 and PF_2 (as in [B]) responses following 5 min, 1 Hz high intensity stimulation to PF_1 during the period shown by the hatched bar, with 50 μM LNARG in the bath and 5 mM BAPTA in the pipette ($n = 5$).

response was evoked. These data indicate that the two inputs were largely discrete. Further to this, the onset of depression in PF_2 was delayed by 1–2 min from that of PF_1 and its rate of development was slower (Figure

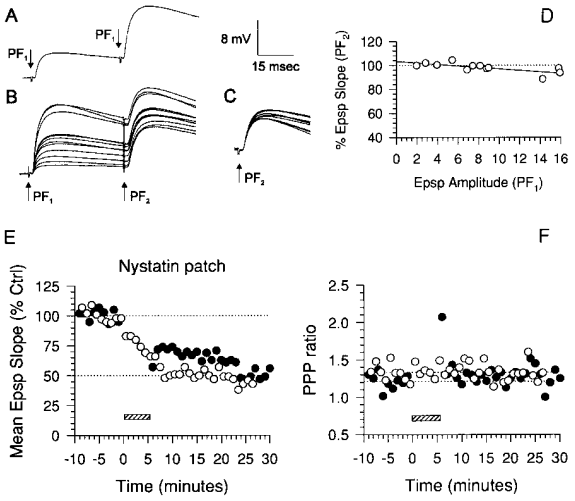

Figure 6. PF_1 and PF_2 Comprise Discrete PF Inputs

(A) Paired pulses applied to PF_1 at a separation of 30 ms.
(B) Over a range of stimulus intensities presented to PF_1, responses to constant strength stimulation of a separate input (50 μm separation, PF_2), at a latency of 30 ms, remained unchanged.
(C) Responses to PF_2 stimulation, taken from (B), are superimposed.
(D) The slopes of PF_2 responses, expressed as percentages of the value obtained at the lowest level of PF_1 stimulation, were independent of the amplitudes of their preceding PF_1 EPSPs.
(E) Effects of 5 min, 1 Hz, high intensity PF stimulation to PF_1 (closed circles) on a cell recorded with the nystatin patch technique. Data shown as in Figure 4B.
(F) The ratio of the second and first responses elicited by paired stimuli at a separation of 30 ms, measured for the same cell shown in (E), are plotted against time.

4B). Finally, depression in PF_2 was largely unaffected by postsynaptic infusion of EGTA or BAPTA, indicating that it was independent of postsynaptic calcium levels (Figures 4B, 4D, and 5A). It was, however, selectively blocked by extracellular perfusion of 20 μM L-nitro-arginine (LNARG), a specific inhibitor of NO synthase (Figure 5B). Depression in PF_1 was largely independent of NO (Figure 5B). Despite these pharmacological differences, both pathways were similarly dependent on a threshold level of stimulation to PF_1 (Figure 5B, open circles).

Given that the NO-mediated depression observed at PF_2 was independent of postsynaptic calcium but generated by stimulation at PF_1, one would have expected a similar, NO-mediated depression to occur at PF_1 in the presence of calcium chelators. To address this issue, the 5 min, 1 Hz high intensity induction protocol was performed on slices perfused with LNARG (50 μM) and with BAPTA (5 mM) included in the recording pipette. Data are summarized in Figure 5C. In accordance with data already shown, depression at PF_2 was completely prevented by LNARG (open circles). Depression of responses at PF_1 (closed circles) not only was prevented, but a strong potentiation emerged that lasted for as long as data could be recorded from the cells (over 90 min). These data suggest, therefore, that NO-mediated depression following high intensity 1 Hz stimulation can occur at both test and control sites but is masked at the test site, in the presence of calcium chelators, by an underlying potentiation.

Pre- or Postsynaptic Origins of LTD_{PF}

Previous reports using microelectrode recording techniques have demonstrated 1 Hz stimulation of PFs to elicit either stable responses (Hartell, 1994b) or a transient potentiation of PF EPSPs (Sakurai, 1990). To investigate whether the whole-cell recording technique employed here, which may disrupt intracellular calcium-buffering mechanisms, itself contributed to the observed depression, perforated patch experiments (Horn and Marty, 1988) were performed. Reasonable access resistances of less than 30 MΩ were obtained within 15–30 min of cell attachment with 220 μM nystatin in the recording pipette. To ensure that the membrane under the patch electrode was not ruptured, calcium green-1 was included in the pipette (0.5 mM). Any cells that were filled with calcium green-1 at the end of the experiment were discarded from the analysis. After a suitable control period, the 5 min, 1 Hz high intensity stimulus paradigm induced depression of responses in PF_1 in 4 out of 6 cells. An example is shown in Figure 6E. In one cell, a strong potentiation emerged following the induction protocol, and in one other, no change was observed. The mean level of depression observed 10 min after the induction protocol (70.4% ± 25.1%, n = 6) was slightly less than that observed with conventional whole-cell recording (61.2% ± 9.0%, n = 5). The extent of depression of responses at PF_2 was, however, statistically indistinguishable from that recorded with conventional whole-cell methods, with all 6 cells undergoing depression to this input (59.7% ± 5.5%, n = 6). These data suggest that while the whole-cell recording technique may facilitate the probability of induction of PF-mediated LTD, presumably by altering the natural calcium-buffering properties of Purkinje cells, it is, by no means, requisite for this form of depression to take place.

High frequency stimulation of PFs has been shown to induce a short-term depression of responses that was attributed to a decrease in presynaptic transmitter levels (Schreurs and Alkon, 1993). A paired pulse protocol was, therefore, used to determine whether depression in either PF_1 or PF_2 had a presynaptic component. Pairs of stimuli were given at 30 ms intervals. Care was taken to ensure that the first response was small enough so that the amplitude of the second did not exceed 7 mV in order to limit the likelihood of calcium entry. Pairing was halted in PF_1 during the induction protocol to ensure that no spiking occurred. Figure 6F indicates, for the same cell illustrated in Figure 6E, that there was no long-term change in the paired pulse ratio of first and second responses in either pathway, following the induction of LTD in either input. Similar results were obtained in all 6 cells using both nystatin (n = 2) and whole-cell recording (n = 4). In 3 of the 6 cells studied, including the example illustrated in Figure 6F, a transient increase in the ratio occurred immediately after the 5 min duration induction protocol. This was limited to PF_1.

Occlusion of LTD_{PF} with LTD_{CJ}

In four cases, LTD induced by raising the frequency and intensity of PF stimulation (LTD_{PF}) was followed by the more conventional protocol of pairing PF stimulation with CF stimulation, 300 times at 1 Hz. Figure 7A illustrates that the second protocol induced little further depression in either pathway. For the collective data, conjunctive stimulation yielded only 15% ± 9% further depression in PF_1 measured after 20 min. In a separate group of 5 cells, conjunctive stimulation of PF and CF inputs induced depression in only PF_1 (33% ± 4% reduction from control; n = 5). Raising the stimulus intensity and frequency to this pathway led to an increase in EPSP amplitude, but to a considerably lesser extent than that of naive cells. No subsequent progressive decline in synaptic strength from this raised level was observed in any of the cells studied with the EPSP slope remaining at 101% ± 3% of the initial increased value after 20 min (for an example, see Figure 7B).

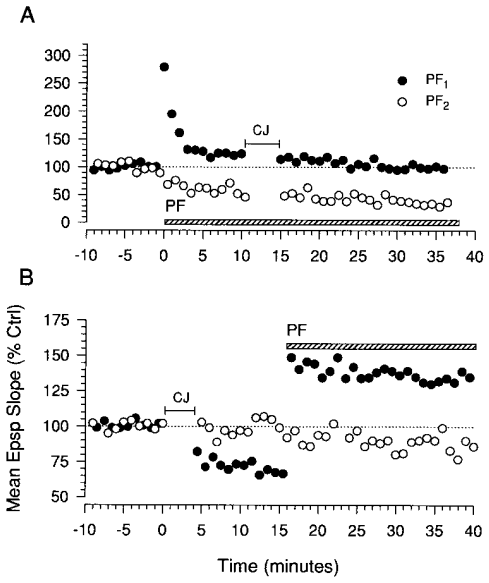

Figure 7. Mutual Occlusion of PF- and CJ-Induced Depression

(A) Synaptic depression in of PF_1 (closed circles), induced by elevating PF stimulus intensity and frequency (PF), largely prevented further depression to the same input by conjunctive stimulation of PF and CF inputs (CJ).

(B) Prior induction of LTD_{CJ} to PF_1 pathway prevented significant depression from the new level following an increase in the stimulus intensity and frequency.

Discussion

Calcium Requirements for LTD_{PF}

PF-mediated LTD was optimally induced in a test PF input to a single Purkinje cell by increasing the frequency of stimulation to 1 Hz, simultaneously with raising the stimulus intensity to yield EPSPs of over 9 mV in amplitude (Figures 5A and 5B). Calcium imaging experiments revealed that precisely the same parameters of stimulation resulted in a gradual accumulation of calcium to levels that approached those following CF stimulation. At lower rates of synaptic activation (0.2 Hz), calcium levels did not accumulate due to the relatively fast rate of recovery to baseline levels (mean decay time constants of 184 ± 68 ms and 524 ± 59 ms). Accordingly,

this lower rate of stimulation was less effective in mediating synaptic depression at this input. At lower stimulus intensities, simply raising the frequency of stimulation had no effect on synaptic transmission, a result that is also predicted by the calcium measurements, since the threshold for calcium influx was not reached. This result suggests that LTD_{PF} does not occur at the single-spine level, since it requires the recruitment of sufficient PFs to exceed the threshold for activation of voltage-gated calcium channels. Together with the finding that LTD_{PF} in PF_1 was prevented by the inclusion of the calcium chelators EGTA or BAPTA in the patch pipette, these data provide strong evidence that PF stimulation alone is capable of mobilizing sufficient calcium to promote synaptic depression.

Several previous studies using a variety of recording techniques, stimulus parameters, and experimental models have failed to detect LTD following PF stimulation alone. Since LTD_{PF} could be demonstrated in cells recorded using the nystatin-perforated patch technique, this form of synaptic depression is not likely to be an artifact of whole-cell recording caused by a disruption of the natural calcium-buffering capabilities of Purkinje cells. Sakurai (1987), using microelectrode recording from Purkinje cell dendrites, reported that 1 Hz stimulation of PFs, in the presence of picrotoxin, led to a temporary potentiation of responses but no depression. Significantly, in that study, EPSP amplitudes were limited to below 7 mV, i.e., subthreshold for the calcium influx predicted here. In other investigations, using both microelectrode and whole-cell patch recording, steady baselines were shown at rates of stimulation of 0.2 Hz or above, even for EPSPs with amplitudes well in excess of the apparent threshold for calcium influx (for example, see Daniel et al., 1992, 1993). In these cases, however, EPSPs were superimposed on a hyperpolarizing current pulse designed to measure the input resistance of the cells. Given the voltage-dependent nature of PF-mediated calcium increases (see Figure 2C; Eilers et al., 1995; cf. Denk et al., 1995), this would effectively reduce the likelihood of calcium influx at a given stimulus intensity.

The level of inhibitory input to Purkinje cells strongly influences the probability of induction of conventional LTD using pairing protocols (Ekerot and Kano, 1985; Schreurs and Alkon, 1993). Activation of inhibitory circuits impinging on Purkinje cells strongly attenuates the level of calcium influx resulting from CF stimulation (Callaway et al., 1995). Furthermore, blockade of inhibition greatly prolongs the duration of PF EPSPs (see Sakurai, 1987). One may surmise, therefore, that in the present report, in which picrotoxin was routinely used, the level of calcium entry resulting from PF stimulation was facilitated. This may explain why, in a previous study performed in the absence of GABAergic block, PF EPSPs of amplitudes above 10 mV remained stable at activation rates of 1 Hz (Hartell, 1994b). Schreurs and Alkon (1993) reported that brief, high frequency stimulation of PFs (100 Hz for 400 ms) led to a short-term depression of PF responses. Despite the strong depolarization that accompanied this stimulation, they suggested that this depression resulted from a presynaptic depletion of transmitter. In the present study, no evidence for a long-term presynaptic component to LTD_{PF} in either test or control pathways was observed. Although the stimulus parameters were very different, it is interesting to speculate that the PF-mediated depression observed by Schreurs and Alkon, in the absence of picrotoxin and using microelectrode recording, occurred through a similar postsynaptic accumulation of calcium to that seen in the present report. A recent report has shown that calcium influx does in fact occur in the absence of picrotoxin or bicuculline following PF stimulation but was restricted to individual or groups of spines (Denk et al., 1995).

Is LTD_{PF} Equivalent to LTD_{CJ}

Two distinct mechanisms of synaptic depression were uncovered. One was calcium dependent, specific to the test site, and did not require NO; the other was diffuse, mediated by NO, but postsynaptically calcium independent. While there is general agreement that calcium is essential for LTD_{CJ} (for review, see Linden, 1994a), a role for NO is controversial. Some authors suggest that NO is required for LTD_{CJ} induction (Shibuki and Okada, 1991; Daniel et al., 1993; Lev-Ram et al., 1995), while others do not (Linden and Connor, 1992; Glaum et al., 1992). There is also debate as to the source of NO: CF (for example, see Shibuki and Okada, 1991; Daniel et al., 1993) or PF (Lev-Ram et al., 1995). Its site of action may well be the Purkinje cell, since the most likely target, guanylyl cyclase, is abundant in the cell bodies, dendrites, and axons of Purkinje cells (Lohmann et al., 1981). In addition, postsynaptic injection of cGMP, the product of guanylyl cyclase, induces LTD when combined with PF stimulation (Daniel et al., 1993; Hartell, 1994b).

It is possible to infer from the present data that strong stimulation of PFs leads to the production of NO, which can then act both locally, in addition to conventional LTD, and at distant sites to induce a form of LTD that does not, seemingly, require the higher rates of PF stimulation normally requisite for LTD induction. The source of NO is unlikely to be the Purkinje cell, since first, NO synthase has not been found in Purkinje cells (Bredt et al., 1991) and second, NO synthase activity is calcium dependent and so NO should not have been produced by the Purkinje cell in the presence of calcium chelators. Depression to the control pathway was clearly observed in cells loaded with high concentrations of EGTA or BAPTA (Figures 4C and 4D). One possible source of NO is the PF itself, and indeed, NO synthase is present in granule cells (Bredt et al., 1991). Other interstitial cells in the molecular layer, including basket cells, also contain NO synthase, and these could well have been activated directly or by strong stimulation of neighboring PFs. The idea that the PF is the source of NO release was recently proposed by Lev-Ram et al. (1995). They demonstrated that release of caged NO could replace PF stimulation to induce LTD in combination with cell depolarization. While the present data support the view that the PF is a likely source of NO, NO-mediated depression in the present study was entirely calcium independent and developed at a much slower rate. Two putative pathways for the NO/cGMP cascade have been proposed: one via calcium-induced calcium release following cyclic ADP–ribose activation of ryanodine-sensitive calcium stores (Berridge, 1993); the other through

phosphorylation of AMPA receptors via a protein kinase G (PKG)-activated phosphatase inhibitor, presumably G substrate (Ito and Karachot, 1992). The former pathway would require calcium as a cofactor (Berridge, 1993) and so is an unlikely mechanism for the depression described here, but it could underlie the rapid depression observed by Lev-Ram et al. (1995). Although this mechanism does not contribute to LTD in cell culture (Linden et al., 1995), neither does the NO/cGMP cascade (Linden and Connor, 1992; Linden et al., 1995). Given this fundamental difference between cultured systems and slices (Crepel and Jaillard, 1990; Shibuki and Okada, 1991, 1992; Ito and Karachot, 1992; Daniel et al., 1993; Hartell, 1994a, 1994b; Lev-Ram et al., 1995; cf. Glaum et al., 1992), a potential role for cADP–ribose dependent in slices should not be excluded. Whether calcium is required for the alternative PKG-mediated phosphorylation pathway is not clear, but the NO-mediated depression described here was blocked, selectively, by the PKG inhibitor KT5823 (data not shown).

Given that the NO-mediated depression was dependent on both the strength and frequency of PF stimulation, it is likely that the extent to which this additional pathway is recruited will be highly dependent on the parameters of PF stimulation. LTD_{CJ}, unlike LTD_{PF}, was found to be input specific (for example, see Figure 7B), in accordance with previous studies both in vivo (Ekerot and Kano, 1985) and in culture (Linden, 1994b). In the present report, stimulus intensities were deliberately minimized, so the threshold for both PF-mediated calcium influx and NO release should not have been reached. If LTD_{CJ} does comprise a mixture of two separate mechanisms, the proportions of each, and hence the degree of input specificity, will depend upon the preparation used and the level of PF stimulation. In cultured cells, for example, little or no contribution would be expected, since first, the number of PF–Purkinje cell synaptic contacts is greatly reduced compared with that in slices and second, this synaptic route is bypassed by direct postsynaptic stimulation with AMPA. Indeed, the NO/cGMP pathway appears not to contribute to LTD in culture systems (Linden et al., 1995).

Prior induction of input-specific LTD by conventional means completely prevented further PF-mediated depression to the same synaptic input. Not only was the ability of the pathway to undergo further change in synaptic strength reduced, the increase in stimulus strength was generally less effective in inducing a large EPSP amplitude, suggesting a decreased sensitivity in this pathway. Following induction of LTD_{PF}, further depression in the same pathway by conjunctive activation of PFs and CFs was clearly reduced compared with that in naive preparations (see Figures 4A and 4B). This mutual occlusion indicates that while each of these forms of plasticity can be pharmacological differentiated, suggesting they run in parallel, they share at least one common intermediate process. The point of convergence is likely to be downstream of both calcium and the NO/cGMP cascade.

Physiological Significance of LTD_{PF}

Although the present data clearly show that PF stimulation alone is capable of inducing a form of calcium-dependent, long-term synaptic depression, it is unclear whether the strong, coordinated, and repetitive activation of groups of PFs that is necessary to induce adequate calcium elevation for LTD_{PF} would occur under physiological conditions. Despite this caveat, examples of cerebellar learning that are neither correlated to nor require CF input have been reported (see Ojakangas and Ebner, 1994; De Schutter, 1995). Alternatively, LTD_{PF} could, in concert with inhibitory inputs, provide a dynamic mechanism that regulates ongoing PF activity to maintain a steady level of Purkinje cell output so that it is maximally responsive to changes in PF inputs. This may be similar to the adaptive filter model of synaptic plasticity proposed in fish hindbrain nuclei (Montgomery and Bodznick, 1994), which have cerebellar-like neural circuits, except for the absence of CFs, and which acts to reduce background noise by recognizing and canceling those inputs that are consistently associated with the animal's own movements.

Recently, De Schutter (1995) proposed that LTD_{PF} could serve as a local negative feedback mechanism to maintain acceptable calcium levels following excessive PF-mediated activation of Purkinje cells. Assuming that the high threshold levels of excitation required to induce PF-mediated calcium influx are met under physiological conditions, the present results provide experimental support for such a neuroprotective hypothesis. However, LTD was not simply restricted to the site of strong stimulation but was spread to distant PF synapses through the actions of NO. This additional NO-mediated mechanism of synaptic depression appears to reinforce the calcium-dependent process by extending the depression to synapses distant from those originally activated, presumably via postsynaptic mechanisms involving cGMP (Daniel et al., 1993; Hartell, 1994). Whether these novel mechanisms of synaptic plasticity underlie a neuroprotective mechanism for Purkinje cells or perhaps an error-independent form of learning and memory remains to be established. They do, however, add a novel mechanism to the function of cerebellar cortical networks.

Experimental Procedures

Experiments were performed on 200 μm thick sagittal slices of cerebellar vermis obtained from 14- to 21-day-old male Wistar rats using standard techniques. Slices were incubated at room temperature in artificial cerebrospinal fluid (ACSF) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 2H_20$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 7H_2O$, 11 mM glucose, equilibrated with 95% O_2 plus 5% CO_2 gas (pH 7.4). Slices were placed between two nylon nets in a chamber mounted on an upright microscope (Nikon Optiphot), fully submerged and perfused with ACSF containing 20 μM picrotoxin at a rate of approximately 2 ml/min. Purkinje cells were visualized using a 40× water immersion lens (0.75 NA), and whole-cell patch recordings were made from the somata of healthy looking cells close to the surface of the slice. ACSF-filled patch electrodes, placed in the granular layer approximately midway between the white matter and a given Purkinje cell, were used to activate CFs. Two additional electrodes were positioned in the molecular layer at a separation of 40–100 μm to activate discrete PF inputs to the same cell. The electrodes were sited equidistant from the soma, at a similar level within the molecular layer, so that they activated regions on opposite sides of the main proximal dendrite.

In current–clamp experiments, cells held at a membrane potential of between −70 and −65 mV patch electrodes, with resistances of between 4 and 5 MΩ, were filled with solutions of the following

composition: 132 mM KCl, 8 mM NaCl, 2 mM $MgCl_2$, 30 mM HEPES, 4 mM Na_2-ATP, 0.3 mM GTP, adjusted to pH 7.3. Depending on the nature of the experiment, EGTA or BAPTA was either omitted and replaced with equimolar KCl or included at a concentration of 5 mM. In some cases, nystatin was included in the internal solution (216 μM). Electrodes were prepared with nystatin-free solution in the tip to facilitate seal formation. Access resistance dropped to levels sufficiently low to permit recording within 15–30 min. For calcium imaging experiments, EGTA/BAPTA was replaced with 1 mM calcium green-1 (Molecular Probes). Shortly after entering whole-cell recording mode, each of the two PF inputs to the cell were activated alternately (200 μs, 5–50 μA, 0.2 Hz). Stimulus intensities were carefully chosen to ensure that the amplitude of the PF EPSPs did not exceed 6 mV during the initial control period to limit the possible entry of calcium through voltage-dependent calcium channels. After approximately 10 min, either the stimulus intensity alone, the frequency, or both the intensity and the frequency of stimulation of just one pathway were raised as described in Results. To ensure that precisely the same stimulus strengths were maintained before and after the induction stimulus paradigm, a separate stimulator was used for the variable strength stimulation. Input and electrode resistances were monitored throughout, and experiments were discarded from the analysis if either of these measurements changed significantly over the course of the experiment. Data were analyzed off-line with a homemade computer program. Unless specifically mentioned, drugs were obtained from Sigma. LNARG was dissolved in water and included in the perfusate throughout experiments and during preincubation.

Fluorescence Measurements

A confocal microscope (Bio-Rad MRC 500) was used to monitor the fluorescence intensity of the calcium indicator calcium green-1. Two types of measurements were made following dye equilibration periods of at least 20 min. Conventional scanning techniques were used to measure relatively long-term changes in fluorescence intensity at high spatial resolution. Full screen images were collected at intervals of between 1 and 15 s over several minutes, and changes in fluorescence intensity, relative to control periods, were calculated. An estimation of relative changes in calcium activity in different regions of the cell was attempted by normalizing the change in fluorescence intensity to the mean prestimulus control intensity (δF/F). Comparisons were made from regions delineated by boxes of the same size that were carefully positioned to enclose completely the regions of interest. Results obtained using this method proved to be similar to those of an earlier investigation (Konnerth et al., 1992) using the dual wavelength indicator Fura-2. Since ratiometric measurements with Fura-2 are independent of dye concentration, this similarity provides some justification that δF/F can yield useful information as to differential changes in calcium concentration within the same cell.

To measure changes in fluorescence intensity with a greater temporal resolution, a line-scanning technique, synchronized to synaptic activation of Purkinje cells, was employed. A single pixel diameter line was positioned over the selected region of interest and was scanned repetitively. In this way, X–T images can be generated that reflect changes in fluorescence intensity at a time resolution of 24 ms. Comparisons were made from different regions of the same cell that fell under the line being scanned and adjacent regions averaged to improve the signal to noise ratio. This technique is limited to approximately 3 s periods of continuous collection. Longer periods of collection are interspersed with gaps during which the data was processed (for example, see Figure 2C).

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